Expression and proteasomal degradation of the major vault protein (MVP) in mammalian oocytes and zygotes

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Abstract

Major vault protein (MVP), also called lung resistance-related protein is a ribonucleoprotein comprising a major part (> 70%) of the vault particle. The function of vault particle is not known, although it appears to be involved in multi-drug resistance and cellular signaling. Here we show that MVP is expressed in mammalian, porcine, and human ova and in the porcine preimplantation embryo. MVP was identified by matrix-assisted laser-desorption ionization-time-of-flight (MALDI-TOF) peptide sequencing and Western blotting as a protein accumulating in porcine zygotes cultured in the presence of specific proteasomal inhibitor MG132. MVP also accumulated in poor-quality human oocytes donated by infertile couples and porcine embryos that failed to develop normally after in vitro fertilization or somatic cell nuclear transfer. Normal porcine oocytes and embryos at various stages of preimplantation development showed mostly cytoplasmic labeling, with increased accumulation of vault particles around large cytoplasmic lipid inclusions and membrane vesicles. Occasionally, MVP was associated with the nuclear envelope and nucleolus precursor bodies. Nucleotide sequences with a high degree of homology to human MVP gene sequence were identified in porcine oocyte and endometrial cell CDNA libraries. We interpret these data as the evidence for the expression and ubiquitin-proteasome-dependent turnover of MVP in the mammalian ovum. Similar to carcinoma cells, MVP could fulfill a cell-protecting function during early embryonic development.

Introduction

Major vault protein (MVP), also called lung resistance-related protein (LRP), is a ribonucleoprotein comprising a major part (> 70%) of the vault particle. The vault particle (Kedersha & Rome 1986) is a flower-like structure composed of two central rings, each surrounded by eight petals (Kedersha et al. 1991). The closed petals form a hollow, barrel-shaped structure with dimensions of approximately 55 x 25–35 nm, thought to be the functional form of the vault. In addition to MVP, the vault particle contains the untranslated, vault RNA of up to 141 bases long (Kic-khoever et al. 1993). Besides the MVP, two other less-represented proteins of 193 and 240 kDa have been purified from the vault particle. There appears to be a single, evolutionarily conserved MVP gene in humans and other mammals (Mossink et al. 2002). In contrast, three distinct MVP isoforms, MvpA, MvpB, and MvpC, have been cloned and identified in Dictyostelium (Vasu & Rome 1995).

The exact function of the vault particle or that of MVP is not known (reviewed by Suprenant 2002, van Zon et al. 2003), though it appears to be involved in multi-drug resistance. MVP is increasingly expressed in certain carcinoma and leukemia cells that are refractory to drug treatment (chemotherapy). It was suggested that the MVP might alleviate the effect of chemotherapeutics on target cells (Schepet et al. 1996). The structure and subcellular localization of the vault particles are consistent with possible function of MVP in the intracellular transport of drugs. However, the deletion of the MVP gene in mouse did not alter the response of the MVP-knockout animals to drug treatment (Mossink et al. 2002). More recently, it was shown that MVP interacts with the activated form of extracellular-regulated protein tyrosine kinase (ERK) signal-regulated, suggesting a role of MVP as a scaffold protein.
in tyrosine-phosphorylation-dependent signaling pathways (Koll et al. 2004). Even in the absence of a complete understanding of MVP function, it can be concluded that MVP is an indicator of cell response to drugs in cancer and possibly in other pathological conditions, providing a valuable clinical tool for predicting the prognosis of cancer treatment (Schefet et al. 2000).

To date, there is only one detailed study describing the expression and distribution of MVP in an animal, the sea urchin embryo (Hami & Suprenant 1997), and one study identifying MVP as one of the proteins present in porcine egg extracts (Novak et al. 2004). To our knowledge, no detailed studies of MVP exist for mammalian embryos, and it is not known whether MVP and the vault particles are present in mammalian embryo or whether they could play any role in mammalian development. We further show that MVP protein, expressed in mammalian ova and zygotes, accumulates in the presence of proteasomal inhibitors. To our knowledge this is the first demonstration that the turnover of MVP is facilitated by the ubiquitin-proteasome pathway. We also show aberrant accumulation of MVP in poor-quality human ova donated by infertility patients and in abnormal porcine zygotes generated by in vitro fertilization (IVF) or somatic cell nuclear transfer (SCNT). These initial findings will allow us to determine whether MVP could play a role in mammalian oogenesis and/or early development, and whether it could be exploited as a potential oocyte/embryo quality marker in assisted reproduction.

Materials and Methods

**Porcine oocyte collection and in vitro maturation**

Detailed procedures for oocyte collection and in vitro maturation have been described previously (Abeydeera et al. 1998, 2000). Briefly, ovaries from pre-pubertal gilts were collected at a local slaughterhouse and transported to the laboratory for the isolation of oocytes for IVF from 2–5 mm antral follicles. Oocytes were also isolated from small antral follicles (<2 mm) for immunodetection of MVP at the germinal-veicle (GV)-stage, but not for IVF. Oocytes with uniform ooplasm and compact cumulus were collected and washed in Hepes-buffered Tyrode’s medium containing 0.01% (w/v) polyvinyl alcohol. The medium used for oocyte maturation was BSA-free tissue-culture medium 199 (Gibco, Grand Island, NY, USA) supplemented with 0.57 mM cysteine, 10 ng/ml epidermal growth factor, 0.5 μg/ml follicle-stimulating hormone, 0.5 μg/ml luteinizing hormone, and 0.1% (w/v) polyvinyl alcohol. Groups of 50 oocytes were matured in 0.5 ml culture medium for 22 h at 38.5°C. The oocytes were then cultured for another 22 h in maturation medium without addition of gonadotropins. At the end of maturation, oocytes were stripped of cumulus cells by vortexing in 0.1% (w/v) hyaluronidase in Hepes-buffered Tyrode’s medium.

**Porcine IVF**

Denuded oocytes were washed three times in TL-Hepes medium and in the fertilization medium, respectively. The fertilization medium was a modified Tris-buffered medium (mTBM) containing caffeine and BSA. 30–35 oocytes were transferred into pre-equilibrated 50 μl drops of mTBM under paraffin oil. Cryopreserved semen was thawed and spermatozoa were washed twice by centrifugation (1000 g for 4 min) in Dulbecco’s PBS (PBS; Gibco) supplemented with 1 mg/ml BSA (Abeydeera & Day 1997). Spermatozoa were resuspended in mTBM, and 50 μl sperm suspension was added to the fertilization drop to give a final concentration of 5 × 10⁵ sperm/ml. Oocytes were co-incubated with spermatozoa for 6 h. Presumptive zygotes were then cultured in North Carolina State University (NCSU)-23 with the addition of 0.4% BSA for the remainder of the experiment. To block proteasome-dependent proteolysis in some experiments, 100 μM MG132 was added to NCSU-23 at the time of washing from spermatozoa (6 h post-insemination).

**Porcine in vivo embryos**

For the collection of in vivo-derived embryos, pre-pubertal gilts received intramuscular injections of 1500IU pregnant mares’ serum gonadotropin (eCG; Intergonan; Intervet America, Millsboro, DE, USA) followed by 5001U human chorionic gonadotropin (Ekluton) 72 h later. At 24 and 36 h after human chorionic gonadotropin, the gilts were inseminated with 3 billion spermatozoa from a fertility-tested boar. Subsequently, the gilts were slaughtered at the local abattoir at 70–74 h post-human chorionic gonadotropin to collect the two–four-cell stage, at 94–98 h to collect the four-cell stage, at 118–122 h to collect the four–eight-cell stage, at 142–146 h to collect the 8–16-cell stage, at 192 h to collect the morula-blastocyst stage, and at 216 h to collect the blastocyst-stage embryos. The oviducts and uteri were flushed, and the embryos were recovered and fixed for immunofluorescence as described below.

**Porcine SCNT**

A day-35 crossbred porcine fetus was obtained from a pregnant gilt. The tissue was cut into small pieces with fine scissors. The cells were incubated for 30 min at 37°C in PBS containing 0.05% trypsin and 0.02 mM EDTA, and then the suspension was centrifuged. The cell pellet was resuspended and cultured in Dulbecco’s modified Eagle’s medium supplemented with 2 mM l-glutamine, 0.1 mM sodium pyruvate, 75 μg/ml penicillin G, 30 μg/ml streptomycin, and 15% (v/v) fetal calf serum. The cells were passaged twice and cultured for 10–13 days before being used as nuclear donors. After 44 h of oocyte maturation, oocytes were freed from cumulus cells by vigorous vortexing for 4 min in TL-Hepes supplemented with 0.1% polyvinyl alcohol and 0.1% hyaluronidase. Cumulus-free
Human oocytes

Human oocytes were obtained from women from infertile couples, undergoing controlled ovarian hyperstimulation and transvaginal oocyte retrieval for infertility as approved by Institutional Review Board, Health Science Section, University of Missouri, Columbia, MO, USA. The etiologies of couples’ infertility included tubal occlusion (two couples) and male factor (one couple). Women underwent typical suppression of gonadotropin-releasing-hormone analogue with follicle-stimulating hormone (FSH)/luteinizing hormone (LH) protocols. Oocytes designated as poor quality by the embryologist at the time of oocyte retrieval were donated for research. Poor quality was defined morphologically including defects in the ooplasm such as darkening, granularity or fractures (Sharpe-Timms & Zimmer 2000). Oocytes were removed from the follicular aspirates, rinsed in PBS, and zona removal and fixation performed as described below for immunofluorescence.

Antibodies and inhibitors

Affinity-purified anti-MVP mouse IgG, purchased from Biogenesis, Kingston, NH, USA (catalog no. 0200-0559; diluted 1/100 for immunofluorescence and 1/1000 for Western blotting), was raised against affinity-purified nuclear extracts of human breast cancer cells of the MCF-7 cell line (Abbondanza et al. 1998). Rabbit anti-ubiquitin serum Ab1690, raised against purified ubiquitin, covalently linked to keyhole-limpet hemocyanin, was purchased from Chemicon (Temecula, CA, USA). Rabbit anti-proteasome serum α/β, purchased from Biomol (US distributor for Affinity Research Products), Plymouth Meeting, CA, USA (catalog no. PW 8155), was raised against proteasomal preparation isolated from human reticulocytes (Tanaka & Tsurumi 1997), and was shown to recognize multiple proteasomal core subunits including subunits α5/α7, β1, β5, β5i, and β7. Rabbit serum for the visualization of embryonic nucleoli (McCauley et al. 2002) was raised against a synthetic peptide corresponding to the C-terminus of ubiquitin-CEP52 tail fusion ribonucleoprotein (Chwetzoff & d’Andrea 1997). Fluorescently conjugated secondary antibodies (goat anti-mouse IgG-FITC (fluorescein isothiocyanate) and -TRITC (tetramethylrhodamine B-isothiocyanate), goat anti-rabbit IgG-FITC and -TRITC) and secondary antibodies for Western blotting were purchased from Zymed (San Francisco, CA, USA). MG132 (Z-Leu-Leu-Leu-CHO), a highly specific, fully reversible inhibitor of proteasomal proteolytic activity (Lee & Goldberg 1998), was purchased from Biomol. MG132 and related inhibitors bind specifically to the 20S proteasomal core via MB1 proteasomal subunit and do not block the activity of non-proteasomal serine proteases, including chymotrypsin, trypsin, and papain (Fenteany et al. 1995, Goldberg et al. 1995).

SDS/PAGE and Western blotting

The oocytes were washed in warm PBS and boiled with Laemmli loading buffer containing 50 mM Tris (pH 6.8), 150 mM NaCl, 20% glycerol, 2% SDS, 5% β-mercaptoethanol, 1 mM PMSF, and 0.01% Bromphenol Blue. Gel electrophoresis was performed in 10% Tris-glycine gels (Cambrex Bio Science, Rockland, ME, USA) using an Owl wet transfer system (Fisher Scientific, Houston, TX, USA) at a constant 50 V for 4 h. The transferred gels, as well as several non-transferred gels, were stained with Coomassie Blue stain and the membranes were processed for Western blotting. The membranes were incubated sequentially with 10% non-fat milk (1 h), mouse anti-MVP antibody (1/2000 dilution, overnight), horseradish peroxidase-conjugated goat anti--mouse antibody (1/10 000 dilution, 1 h), and chemiluminescent substrate (SuperSignal; Pierce, Rockford IL, USA). The membrane was used to expose Kodak BioMax Light Film (Kodak, New Haven, CT, USA) for 1 min using a Kodak M35A X-OMAT Processor (Kodak). Densitometry was performed by the Kodak Electrophoresis Documentation and Analysis System 290 (EDAS 290) with image capture by the Kodak DC 290 camera. Image analysis was performed by Kodak 1D Image Analysis software (Kodak Scientific Imaging Systems, New Haven, CT).

For reprobing the membranes with anti-ubiquitin antibody, the membranes were incubated with stripping buffer (62.5 mM Tris, pH 6.8, 100 mM β-mercaptoethanol, and 2% SDS) at 56°C for 30 min. After thoroughly washing and blocking, they were probed with anti-ubiquitin antibody, AB1690 (1/2000 dilution), following the standard method. Films were scanned and relative densities of visible bands were measured using Kodak densitometry system. For the isolation of ubiquitinated ooplasmic proteins, 300 metaphase-II ova were lysed and extracted as described above, and incubated with recombinant, agarose-matrix-bound ubiquitin-binding protein p62 (catalog no. UW9010; Biomol). Following incubation at room temperature for
20 min., complexes of p62 and ubiquitinated ooplasmic proteins were eluted in SDS/PAGE loading buffer. Ubiquitinated proteins were resolved on 5–20% reducing gel, transferred on to PVDF membrane and probed with anti-MVP and anti-ubiquitin antibodies as described above.

Matrix-assisted laser-desorption ionization-time-of-flight (MALDI-TOF)

Porcine zygotic proteins were separated electrophoretically on one-dimensional, SDS/PAGE (10% gels) and transferred to PVDF membrane as described for Western blotting. After transfer, the gel with the retained and the prominent triplet of bands at 100–105 kDa was stained with Methylene Blue and rinsed with buffer. The top and bottom bands within the triplet were excised carefully using a sterile blade and transported in buffer to the Proteomics Core of University of Missouri, Columbia, MO, USA. The excised bands were processed separately. Each band was digested with trypsin and the digests were desalted on prepared C18 ZipTips. Bound peptides were eluted in 10 μl from ZipTips with acetonitrile/water/88% formic acid (700:290:10; by vol.). The eluted peptides were analyzed by MALDI-TOF MS (Applied Biosystems Voyager System 6266). Spectra were submitted to Protein Prospector and searched against the NCBI database.

cDNA libraries

Porcine oocyte (Whitworth et al. 2003), endometrium, and ovary cDNA libraries (Jiang et al. 2001) were constructed as described and approximately 2000 clones were randomly sequenced per library.

Immunofluorescence

Basic procedures and solutions were described previously (Sutovsky et al. 2003, Sutovsky 2004). Ova and embryos were released from zona pellucida by short (1–2 min) incubation in TALP-Hepes medium with 0.5% pronase, fixed in 2% formaldehyde in PBS, and permeabilized in 0.1% Triton X-100 in PBS. Blocking was performed by 30 min incubation with PBS containing 5% normal goat serum and 0.1% Triton-X-100. Antibodies were diluted and washes were performed in a labeling buffer composed of 0.1 M PBS with 0.1% Triton-X-100 and 1% normal goat serum. First antibody was a mix of anti-MVP mouse IgG (1/100), sometimes combined with a rabbit serum against proteasomal subunit α/β (1/200), ubiquitin (AB1690; 1/100) or ubiquitin-CEP52 tail fusion ribonucleoprotein (1/100). After a wash, the primary antibodies were detected by a mixture of goat anti-mouse IgG-FITC, goat anti-rabbit IgG-TRITC, and DNA stain DAPI (4,6-diamidino-2-phenylindole), all three diluted 1/80. Both primary and secondary antibody incubations were carried out for 40 min. Negative controls were performed by the incubation of ova and embryos with non-immune rabbit and mouse sera (purchased from Sigma) at the concentrations identical to those of specific antibodies listed above. Examples of such negative controls are shown in Fig. 5i and 5j. Multiple trials were performed with GV/metaphase-II stage ova, IVF-generated zygotes and embryos, in vivo zygotes and embryos, and embryos generated by SCNT. Porcine oocyte and embryo numbers and MVP staining patterns are shown in Table 1. Human ova were obtained from three consenting patients and processed in four separate trials. Human oocyte numbers and staining patterns are shown in Table 2.

Results

As a part of our ongoing effort to evaluate the role of the ubiquitin-proteasome pathway in preimplantation embryonic development, we conducted a biochemical and proteomic comparison of proteins that accumulate in porcine zygotes under conditions prohibitive of proteasomal protein degradation. We treated the inseminated porcine

Table 1 Summary of MVP-staining patterns in porcine ova, zygotes, and embryos processed for immunofluorescence.

<table>
<thead>
<tr>
<th>Stage and system</th>
<th>Sum ova (100%)</th>
<th>Diffuse, granular ooplasmic staining</th>
<th>MVP-positive aggregates/vesicles (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Germinal vesicle</td>
<td>30</td>
<td>28</td>
<td>2 (6.7)</td>
</tr>
<tr>
<td>Metaphase II</td>
<td>16</td>
<td>16</td>
<td>0 (0)</td>
</tr>
<tr>
<td>In vitro</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 cell</td>
<td>15</td>
<td>14</td>
<td>1 (6.7)</td>
</tr>
<tr>
<td>2–4 cell</td>
<td>17</td>
<td>11</td>
<td>6 (35.3)</td>
</tr>
<tr>
<td>8–cell morula</td>
<td>12</td>
<td>10</td>
<td>2 (16.6)</td>
</tr>
<tr>
<td>Blastocyst</td>
<td>16</td>
<td>10</td>
<td>6 (37.5)</td>
</tr>
<tr>
<td>In vivo</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 cell</td>
<td>6</td>
<td>6</td>
<td>0 (0)</td>
</tr>
<tr>
<td>2–4 cell</td>
<td>6</td>
<td>6</td>
<td>0 (0)</td>
</tr>
<tr>
<td>8–cell morula</td>
<td>7</td>
<td>6</td>
<td>1 (14.3)</td>
</tr>
<tr>
<td>Blastocyst</td>
<td>11</td>
<td>7</td>
<td>4 (36.4)*</td>
</tr>
<tr>
<td>SCNT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 cell</td>
<td>23</td>
<td>21</td>
<td>2 (8.7)</td>
</tr>
<tr>
<td>2–4 cell</td>
<td>11</td>
<td>9</td>
<td>2 (18.2)</td>
</tr>
<tr>
<td>8–cell morula</td>
<td>6</td>
<td>5</td>
<td>1 (16.7)</td>
</tr>
<tr>
<td>Blastocyst</td>
<td>12</td>
<td>6</td>
<td>6 (50)</td>
</tr>
<tr>
<td>Total ova</td>
<td>188</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* None of these in vivo blastocysts displayed aberrant morphology of trophoblast or inner cell mass.
ova with a specific proteasomal inhibitor MG132 at 6 h after gamete mixing in vitro, to avoid the block of the proteasome-dependent step in zona penetration by the sperm (see Sutovsky et al. 2003, 2004). We harvested the fertilized ova at 24 h after insemination for proteomic analysis and resolved the protein extracts on one-dimensional SDS/PAGE.

Among the bands that appeared de novo in the fertilized ova exposed to MG132, the most prominent was a triplet of closely adjacent protein bands, migrating on Coomassie Blue-stained gels (100 ova/lane) within the approximately 100–105 kDa range (Fig. 1A). This band triplet became even more prominent after the transfer of proteins from SDS/PAGE gel to a PVDF membrane for Western blotting, since a major portion of it consistently remained untransferred on the gels in three separate trials (Fig. 1B). Incomplete transfer of these MVP bands was consistently seen in repeated experiments with varied transfer conditions and protein membranes. Two peripheral bands (top and bottom bands within the triplet) were excised from the gel after protein transfer and sequenced separately using MALDI-TOF. The micro-sequencing of the trypsin-digested peptides from these two distinct bands yielded identical results, i.e. identification of both bands as the MVP (Fig. 2). The identification of both bands was highly accurate, with even coverage of protein sequences with the identified peptide fragments (Fig. 2).

Western blotting with a commercial, thoroughly characterized mouse monoclonal antibody (IgG) against human MVP confirmed the identity of the 100–105 kDa band triplet in the MG132-treated, fertilized ova as the MVP (Fig. 1C). Collectively, the relative density (RD) of all MVP

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Table 2 Summary of MVP-staining patterns and oocyte-quality grades in human ova processed for immunofluorescence with anti-MVP antibodies. Oocyte classification at retrieval according to Sharpe-Timms & Zimmer (2000) was applied as the following grades: (4) excellent; (3) good; (2) fair; (1) poor. An additional nine ova disintegrated during processing and are not included in the analysis.

<table>
<thead>
<tr>
<th>Trial no.</th>
<th>Number of ova</th>
<th>Grade of ova</th>
<th>MVP-staining pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>1</td>
<td>Diffuse MVP in ooplasm</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>1</td>
<td>MVP-positive vesicles in ooplasm</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>1</td>
<td>Diffuse MVP in ooplasm and accumulation in polar body</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>1,1,1,1</td>
<td>Diffuse MVP in ooplasm and accumulation in polar body</td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 1 Proteomic identification of MVP in the porcine zygotes. (A) Coomassie Blue-stained SDS/PAGE gel before protein transfer to a PVDF membrane. Lane 1, molecular-mass standards; lane 2, 100 zygotes cultured for 24 h under control conditions; lane 3, 100 zygotes culture until 24 h post-insemination in the presence of 100 μM MG132 (added at 6 h post-insemination). Arrowheads point to the bands only seen in the MG132-treated zygotes. (B) Coomassie Blue staining of an SDS/PAGE gel after protein transfer to a PVDF membrane. Lane 1, 100 metaphase-II ova; lane 2, 100 zygotes after 24 h of culture under control conditions; lane 3, 100 zygotes cultured until 24 h post-insemination in the presence of 100 μM MG132 (added at 6 h post-insemination). Lane 3 contains a prominent triplet of bands in the 100–105 kDa range. This triplet was excised from the gel shown here and identified as being the MVP bands by MALDI-TOF peptide microsequencing (see Fig. 2). (C) Western blot of 100 porcine ova/zygotes per lane, probed with mouse monoclonal anti-MVP antibody. Lane 1, 100 metaphase-II ova; note the presence of a unique band of approximately 179 kDa. Lane 2, 100 zygotes cultured until 24 h post-insemination in the presence of 100 μM MG132 (added at 6 h post-insemination); note the increased density of the 100–105 kDa bands and several unique bands in the 49–100 kDa range, likely the products of non-proteasomal degradation of MVP. Lane 3, 100 zygotes after 24 h of culture under control conditions. Combined RD (× 10^6) of all visible bands is shown below individual lanes. (D) Lane 1 from the protein membrane corresponding to lane 1 of Western blotting film shown in (C). The membrane was stripped and re-probed with anti-ubiquitin antibody AB1690. The band of approximately 179 kDa (arrow) is labeled prominently, corresponding to an MVP-immunoreactive band of approximately 179 kDa in lane 1 of (C). (E) Western blot of ubiquitinated protein preparations affinity-purified from 300 metaphase-II-arrested ova using recombinant ubiquitin-binding protein p62 immobilized on agarose matrix (lane 1). Only the polyubiquitinated, high-mass MVP band (arrow) is present. After stripping, this MVP band is within the range of high-molecular-mass smear of various polyubiquitinated substrates (vertical arrow) commonly recognized by anti-ubiquitin antibody AB1690 (lane 2), which also recognizes the presumed monoubiquitin band of 8.5 kDa (lane 2, *). (F) Negative control probed with non-immune sera and appropriate secondary antibodies.
bands in the MG132-treated zygotes (RD = 18.5 \times 10^6) was 2.5 times higher than in metaphase-II ova (RD = 7.4 \times 10^6) and nearly three times higher than in the fertilized ova not exposed to MG132 (RD = 6.6 \times 10^6). It should also be considered that the accumulation of MVP bands in the presence of a proteasomal inhibitor would be even more pronounced if all MVP bands in the MG132 lane were transferred completely from SDS/PAGE gels. This suggests that the block of proteasomal activity caused the accumulation of MVP and that MVP content of mammalian ova may be reduced by proteasomal proteolysis after fertilization (Fig. 1C). Consistent with proteasomal degradation of MVP, Western blotting revealed a single high mass (approximately 179 kDa) band immunoreactive to MVP in the non-fertilized, metaphase-II-arrested ova (Fig. 1C). After stripping and re-probing of the PVDF membrane, this approximately 179 kDa band was the most prominent band immunoreactive to anti-ubiquitin antibodies (Fig. 1D). This MVP band was also detected by anti-MVP antibody on Western blots of ubiquitinated proteins.

Figure 2 Amino acid sequence of the human MVP marking the peptides present in the extracts of porcine ova treated with MG132, as identified by MALDI-TOF MS peptide sequencing. The top and bottom bands from the triplet of bands migrating in the 100–105 kDa range on one-dimensional SDS/PAGE were excised, digested with trypsin, and subjected to MALDI-TOF peptide sequencing. Matched amino acid sequences are underlined for the top band and shaded for the bottom band. Sequences are matched against the human MVP/LRP sequence (accession no. CAA56256). Note the even coverage of the sequence with matched peptides, and many close/exact peptide matches between the two respective bands.

Figure 3 Identification of the partial MVP sequences in the cDNA libraries prepared from porcine GV ova (178 bp; sequence no. pggv0-014-g09) and porcine endometrium (345 bp; sequence no. pd6end2-007-h07). The expressed sequence tags were identified by blasting to Tigr-\textit{Sus scrofa} database maintained by the Institute for Genomic Research.
protein preparations affinity-purified from metaphase-II-arrested ooplasm using recombinant ubiquitin-binding protein p62 immobilized on agarose matrix (Fig. 1E, lane 1). After stripping, this MVP band was within the high-molecular-mass smear recognized by anti-ubiquitin antibody AB1690, which also recognized the presumed monoubiquitin band of 8.5 kDa (Fig. 1E, lane 2). As expected, the nascent, non-ubiquitinated 100–105 kDa MVP species was not present. The ubiquitin-immunoreactive MVP band was not prominent in the fertilized ova with or without MG132 treatment, while all types of ova contained at least one MVP band in the 100–105 kDa

Figure 4 Immunofluorescence of MVP (green) in porcine ova, zygotes, and embryos generated by IVF and embryo culture. Typical, representative patterns are shown. DNA was counterstained with DAPI (blue) and superimposed over the corresponding light-microscopic images acquired with differential interference-contrast (DIC) optics, lettered with a prime ('). (A) GV-stage ovum from a small antral follicle. Inset shows the accumulation of the MVP around the nucleolus. (B) GV-stage ovum from a large antral follicle. Accumulation of the MVP is visible around the nuclear envelope (arrowheads). (C) Metaphase-II oocyte with a metaphase plate of chromosomes on the left side; note the accumulation of MVP in the polar body on the right side. (D) Oocyte—cumulus complex containing a metaphase-II-stage ovum. MVP accumulation is prominent in the ooplasm, but not in the surrounding cumulus cells. (E) Pronucleate stage zygote, 20 h post-insemination. (F) Two-cell embryo; note an aggregate of MVP in the left blastomere. (G—I) Four-, eight, and 16-cell embryos, respectively. (J) Blastocyst with patches of MVP-immunoreactive proteins. (K) Defective, fragmented early embryo showing distinct MVP aggregates. Scale bars, 10 μm.
range. Two degradation products migrating slightly above the 49 and 77 kDa markers were present in the lysates of unfertilized ova, and more prominently in both the control and the MG132-treated zygotes. The MG132-treated zygotes also contained several unique bands between 77 and 105 kDa (Fig. 1C). Such a multi-band pattern is expected for MVP due to its biochemical properties and proteolytic degradation (see the Discussion). None of the above bands were revealed in control blots with non-immune mouse serum (Fig. 1F).

Further to MALDI-TOF peptide sequencing and Western blotting identification of MVP, the appropriate partial sequences (Fig. 3) were found in the cDNA libraries prepared from porcine GV-stage ova (178 bp; sequence no. pgvo4-014-g09) and porcine endometrium (345 bp; sequence no. pd6end2-007-h07). These expressed

Figure 5 Representative immunofluorescence images of MVP (red) in porcine ova, zygotes, and embryos generated in vivo. DNA was counterstained with DAPI (blue) and superimposed over the corresponding light-microscopic images acquired with differential interference-contrast (DIC) optics, lettered with a prime ('). The following stages are shown: (A) four-cell, (B) eight-cell, (C) 16-cell (D) morula (E) early blastocyst, (F) detail of an early blastocyst, (G) blastocyst with a well-differentiated inner cell mass (arrowheads) showing the accumulation of the MVP, (H) blastocyst showing MVP aggregation in apoptotic blastomeres, and (I, J) negative controls at four-cell and morula stages, respectively. Scale bars, 10 μm.
Figure 6 Immunofluorescence of MVP (green) in porcine ova, zygotes, and embryos generated by SCNT. (A) Normal SCNT zygote at 24 h with a remodeled donor-cell nucleus after successful nuclear transfer and activation. Inset shows a single nucleus with a high concentration of proteasomes (red; proteasomal core subunits of types α and β). (B) Accumulation of the MVP in a control SCNT zygote at 24 h after nuclear transfer and activation. (C, D) MVP distribution and the incomplete remodeling of the donor nucleus (C’, inset) in a reconstructed porcine zygote cultured in the presence of 10 μM MG132 for the first 24 h after SCNT. (E) Absence of donor cell nuclear remodeling in a porcine ovum cultured in the presence of high concentration, 100 μM, of MG132 for the first 24 h after SCNT. Note the perinuclear accumulation of MVP (E’, inset). (F) Control, two-cell-stage embryo generated by SCNT without MG132 treatment. Note the aggregation of MVP in the cytoplasm of both blastomeres and a karyomere present in the left blastomere (F’, inset; red labeling represents proteasomal subunits of types α and β). (G) SCNT blastocyst, day 6. (H) A hatching SCNT blastocyst, day 7. (I) Accumulation of the MVP in an apoptotic blastomere of an otherwise normal SCNT blastocyst. Nucleolar ribonucleoprotein CEP52 is counterstained red (I’) and shows the restricted nucleolar localization in healthy blastomeres surrounding the apoptotic one. (J) Cytoplasmic accumulation of MVP (green) within the distinct cytoplasmic vesicles/aggregates in a fragmented one-cell stage SCNT embryo. DNA was counterstained with DAPI (blue) and superimposed over the corresponding light-microscopic images acquired with differential interference-contrast (DIC) optics, lettered with a prime (’). Scale bars, 10 μm.
sequence tags were identified by blasting to Tigr-Sus scrofa database maintained by the Institute for Genomic Research (http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=pig). The highest score for human MVP/LRP homologue was 295.

By immunofluorescence (Table 1), MVP showed a predominantly cytoplasmic distribution in normal porcine ova at the GV, metaphase-II, one-cell, two-cell, 8–16-cell, morula, and blastocyst stages of preimplantation development in vitro (Fig. 4) and in vivo (Fig. 5). Typically, the MVP was concentrated in a form of fine particles, presumably the vaults, around the large, round cytoplasmic inclusions and membrane profiles typical of porcine ova (Fig. 4A; see also Figs. 5F, 6B, and 6C). The in vitro-derived blastocysts also showed distinct accumulation of MVP in the inner cell mass (Fig. 5G). This was not observed in the in vitro-derived blastocysts since they typically had a low cell count and did not form a discernible inner cell mass. Some GV ova, zygotes, and embryos at various stages of development in vitro and in vivo displayed the accumulation of MVP-containing particles around the nuclear envelope (e.g. Fig. 4B) and around the nucleolus precursor bodies (e.g. Fig. 4A). Such distribution is consistent with the localization of MVP in the sea urchin embryo (Hamill & Suprenant 1997) and in other cell types (Chugani et al. 1993). Cytoplasmic accumulation of the MVP in distinct patches and vesicle-like aggregates was observed in some of the abnormal IVF embryos, especially in those that underwent fragmentation during in vitro culture (e.g. Fig. 4K). Such MVP-immunoreactive patches were also observed frequently in the in vitro-generated blastocysts (e.g. Fig. 4I). The in vitro-fertilized ova, cultured for 26 h in the presence of 10 and 100 μM MG132, were also screened. The accumulation of MVP, due to the obliteration of proteasomal activity, was detectable using biochemical techniques (see Fig. 1) but, apart from somewhat increased cytosolic fluorescence, it did not produce a distinct accumulation pattern by epifluorescence light microscopy.

The SCNT procedure resulted in production of zygotes with one large pronucleus and a typical cytoplasmic distribution of MVP (Fig. 6A and 6B; 24 h post-SCNT), while some zygotes with deviant pronuclear organization (two pronuclei, multiple karyomeres) were also produced (results not shown). In contrast to in vitro-fertilized ova, the treatment with MG132 had a profound effect on nuclear morphology and MVP distribution in the SCNT zygotes. At 10 μM, MG132 applied immediately after SCNT and maintained in the embryo culture medium for 24 h caused either premature chromatin condensation or aberrant, incomplete remodeling of the donor cell nucleus. Multiple patches of condensed heterochromatin, and an increased accumulation of MVP around the cytoplasmic Golgi profiles, were seen frequently (Fig. 6C and 6D). Nuclear labeling of the protosomal subunits suggested that the proteasomes did at least in part infiltrate these nuclei (Fig. 6C). The ova cultured for the first 24 h after SCNT in the presence of 100 μM MG132 showed no nuclear remodeling. The intact donor-cell nuclei were often surrounded by patches of MVP-immunoreactive material, possibly a result of failed MVP import through the nuclear envelope, or a failure of nuclear-envelope reconstitution due to cell-cycle blockage brought about by MG132 (Fig. 6E).

Aberrant accumulation of the MVP was also observed in some of the control SCNT ova (no MG132 exposure) that developed beyond the one cell stage (Fig. 6F–6J), and in the apoptotic blastomeres in many of the SCNT-blastocysts (Fig. 6I). Porcine embryos are produced routinely by both IVF and SCNT methods described here and are capable of implantation and development to term after embryo transfer (Macháty et al. 1998, Lai et al. 2001, Lai & Prather 2003). If allowed to develop to the blastocyst stage in vitro, they have a low blastomere count and poor morphology not comparable to the in vivo-generated blastocysts (see Fig. 5). It is thus not surprising that the patterns of MVP distribution were different between the in vitro- and the in vivo-generated blastocysts.

The imaging analysis of porcine ova and embryos from various culture systems (Table 1), excluding the SCNT zygotes exposed to MG132 (aberrations described above), can be summarized as follows. An exclusively diffuse, finely granulated cytoplasmic labeling is a prevailing pattern in all categories in vitro, in vivo and following SCNT.

The granulated ooplasmic pattern is reflective of the assembled vault particles and was most prominent in all fertilized categories in vivo and in vitro, as well as in the SCNT zygotes. Accumulation of MVP in the cytoplasmic vesicles was seen in some of the GV-stage ova, but in none of the metaphase-II ova. This pattern was also frequent in the in vitro and SCNT embryos from zygote up to the morula stage. Interestingly, a large portion of blastocysts in all three systems (in vivo, in vitro and SCNT) contained at least one blastomere with MVP-positive vesicles and a fragmented nucleus indicative of apoptosis. This is likely a result of naturally occurring apoptosis found at a low rate within morphologically normal blastocysts. Blastocysts with grossly abnormal morphology and a large number of apoptotic cells were only observed in the IVF and SCNT groups and not in the in vivo group.

Cytoplasmic vesicles containing the aggregates of MVP were also observed in poor-quality human ova (Fig. 7A–7D) donated by women undergoing infertility treatment (Table 2). Besides MVP, these cytoplasmic vesicles also showed immunoreactivity with anti-ubiquitin (Fig. 7B) and for the proteasomal core-subunits of types α and β (Fig. 7C). These MVP-containing aggregates in human ova were comparable in size and appearance to the vesicles/ aggregates seen in the abnormal porcine ova and zygotes (Fig. 7E).
The present data indicate that the turnover of the MVP in the mammalian ovum is regulated by the ubiquitin-proteasome pathway. This omnipresent proteolytic system ensures rapid, substrate-specific degradation of various proteins in the cell cytoplasm and nucleus (reviewed by Pickart 1998, Glickman & Ciechanover 2002). Ubiquitin, an evolutionarily conserved chaperone protein of 8.5 kDa, is transcribed from multiple polyubiquitin genes, translated as a polymer and edited into single molecules by the action of specific ubiquitin-C-terminal hydrolases. Single ubiquitin molecules are activated by ubiquitin-activating enzymes (UBAs; E1 enzymes) and the C-terminal glycine residue of the activated ubiquitin is covalently ligated to the substrate’s internal lysine residue with the help of ubiquitin-conjugating enzymes (UBCs) including ubiquitin carrier protein (E2), ubiquitin-protein ligases (E3s), and sometimes ubiquitin chain-elongation factor E4 (Glickman & Ciechanover 2002). Additional ubiquitin molecules can be ligated to one of the seven internal lysines within the substrate-bound ubiquitin molecule, to form di-, tri-, tetra-, or polyubiquitin chains. Each ubiquitin molecule within such a chain adds 8.5 kDa to the molecular mass of the ubiquitinated substrate. A chain of four or more ubiquitins targets the ubiquitinated substrate to the 26 S proteasome. The proteasome is a holoenzyme typically composed of a barrel-shaped 20 S core, capped with one or two 19 S regulatory complexes or an 11 S activator complex. The 20 S core of the proteasome is composed of four

Discussion

The present data indicate that the turnover of the MVP in the mammalian ovum is regulated by the ubiquitin-proteasome pathway. This omnipresent proteolytic system ensures rapid, substrate-specific degradation of various proteins in the cell cytoplasm and nucleus (reviewed by Pickart 1998, Glickman & Ciechanover 2002). Ubiquitin, an evolutionarily conserved chaperone protein of 8.5 kDa, is transcribed from multiple polyubiquitin genes, translated as a polymer and edited into single molecules by the action of specific ubiquitin-C-terminal hydrolases. Single ubiquitin molecules are activated by ubiquitin-activating enzymes (UBAs; E1 enzymes) and the C-terminal glycine residue of the activated ubiquitin is covalently ligated to
concentric rings containing seven proteasomal subunits of the α-type (two outer rings) and seven subunits of the β-type (two inner rings). The ubiquitinated protein is typically docked to the 19S cap where it is deubiquitinated and transported to the lumen of the 20S core in which the substrate protein is cleaved into small oligopeptides of 3–23 amino acids. Both the ubiquitination and the proteasomal degradation are ATP-dependent.

Multi-band electrophoretic migration patterns of MVP species in control ova and MG132-treated zygotes can be explained by the biochemical properties of the MVP protein and by the mechanism of MG132 action respective to the topology of substrate–protein deubiquitination in the proteasomal regulatory complex and proteolytic cleavage in the proteasomal core. The high-molecular-mass band (approximately 179 kDa), present in the lysates of unfertilized ova, showed immunoreactivity with anti-ubiquitin antibodies and affinity to ubiquitin-binding protein p62, indicating that it was a polyubiquitinated species of MVP. This band was not present in the fertilized zygotes, possibly as a result of increased protein turnover and increased proteasomal activity in the zygotic cytoplasm after oocyte activation and resumption of the cell cycle. One result of such a fertilization-induced increase in proteasomal activity is seen during the degradation of paternal mitochondria after fertilization in pigs and other species (Sutovsky et al. 2000, 2003). This increase in proteasomal activity is in accordance with overall metabolic and genomic activation of the dormant oocyte shortly after fertilization (reviewed by Epel 1990, Latham 1999), and also with the early transcription of proteasomal subunit genes after fertilization (Hamatani et al. 2004). The treatment of the fertilized ova with MG132 did not prevent the disappearance of this 179 kDa MVP band, while the accumulation of MVP bands at and below 105 kDa was seen. This MVP accumulation was reflected by a 2.5-fold increase in the combined RD of MVP bands on Western blots of the lysates of MG132-treated ova, and because the MVP was not present in the fertilized zygotes, compared with metaphase-II ova cultured without MG132. A possible explanation for this pattern of MVP accumulation rests in the proteasomal degradation are ATP-dependent.

In complete transfer of the MVP bands from gels to PVDF membranes (see Fig. 1B) is probably due to the large quantity of this particular protein being present in the lysates of MG132-treated ova, and because the MVP has a long (about 150 amino acids) α-helical domain near its C-terminus and essentially behaves as a hydrophobic molecule (Herrmann et al. 1996). Electrophoretic migration in three distinct, closely adjacent bands at the 100–105 kDa range could be a result of hyper-phosphorylation. The MVP protein contains multiple phosphorylation sites for various protein kinases including protein kinase C, CK-II, and tyrosine kinases (Herrmann et al. 1996, Kolli et al. 2004). Consequently, a similar multi-band pattern arises after the phosphorylation of MVP in vitro (Herrmann et al. 1996).

The exact function of MVP/LRP or that of the vault particle is not known. It has primarily been implicated in multi-drug resistance, a major cause of the failure of cancer treatment (reviewed by Scheffer et al. 2000, Izquierdo et al. 1996). Subsequently, the MVP is regarded as an adverse prognostic factor for chemotherapy. Several functions of the vault particle have been proposed, including intracellular transport, assembly of ribonucleoprotein particles, and proteolytic degradation of ribonucleoproteins (reviewed by Suprenant 2002). Also suggestive of MVP's importance is the observation that the mutation of multiple MVP genes in Dictyostelium impedes cell growth under nutritional stress (Vasu et al. 1993, Vasu & Rome 1995). Recent studies show that mammalian MVP is a substrate of ERKs and tyrosine phosphatase SHP-2 (Kolli et al. 2004). The authors suggested that MVP may serve as a scaffold protein in tyrosine-phosphorylation-dependent signaling pathways. This is consistent with our data showing multi-band pattern suggestive of MVP phosphorylation after in vitro fertilization and culture in the presence of MG132. Phosphorylation may also be a signal for the degradation of MVP after fertilization, as substrate phosphorylation is a major factor in substrate recognition by ubiquitin-conjugating enzymes (reviewed by Glickman & Ciechanover 2002).

Our data show that the levels of MVP, measured by band densitometry after Western blotting, were reduced after fertilization and increased by MG132 treatment of the fertilized ova. This is consistent with the ubiquitination of MVP in the metaphase-II-arrested ova and with its accelerated degradation after fertilization. This MVP proteolysis could be a result of targeted protein turnover in the fertilized ova, or a consequence of developmentally programmed degradation of stored maternal proteins during oocyte-to-embryo transition, as proposed recently for other ubiquitinated maternal proteins in the invertebrate zygote (DeRenzo & Seydoux 2004). The accumulation pattern of MVP in the porcine zygotes treated with...
inhibitors of proteasomal protein degradation is reminiscent of MVP accumulation found in poor-quality human oocytes, and in the abnormal porcine zygotes and embryos generated by IVF and SCNT. This indicates that aberrant oogenesis and embryonic development prior to implantation could be either a cause or a result of a reduced proteolytic capacity of the resident ubiquitin-proteasome system. Altogether, it is plausible that MVP expression could be both a good indicator of human oocyte quality and a sensitive gauge of epigenetic effects induced by hormonal stimulation of patients in vivo, and by culture media and additives in vitro. Further studies of higher-quality human oocytes are warranted; however, invariably the women donating their ova for research undergo controlled ovarian hyperstimulation with injectable gonadotropins and produce multiple ova with the potential for reduced competence for meiotic maturation. The present studies of the mammalian embryonic MVP also provide the necessary background for addressing a possible role of the MVP in early embryo development. Although the MVP-deficient mouse oocytes matured, fertilized and cultured in vitro of pig oocytes in protein-free culture media: fertilization and subsequent embryo development in vitro. Biology of Reproduction 58 1316–1320.


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